Original article

Genotoxicity evaluation of aqueous extracts of Cotoneaster discolor and Alhagi pseudalhagi by comet assay

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BACKGROUND: Cotoneaster discolor and Alhagi pseudalhagi are two important kinds of Iranian manna. They have several therapeutic applications in adults and children. The most important ingredient of Cotoneaster discolor is mannitol. Due to the scarcity of toxicological studies on these compounds, their genotoxicity was evaluated. **METHODS**: Comet assay technique using fluorescence microscopy was selected to assess genotoxicity. Tail length, %DNA in tail, and tail moment were measured and DNA damage was evaluated. **RESULTS:** Our findings showed that A. pseudalhagi, C. discolor, glucose, and mannitol caused DNA damage at concentrations of 5 µg/ml, 100 mg/ml, 25 mg/ml, and 50 mg/ml, respectively. **CONCLUSIONS:** Taking C. discolor and A. pseudalhagi in doses which produce concentrations less than 100 mg/ml and 5 µg/ml, respectively, is safe. The harmful effects of non sugary components might be considered in the toxicity caused by these compounds.

KEYWORDS: Alhagi Pseudalhagi, Cotoneaster Discolor, Genotoxicity, Comet Assay

BACKGROUND

Consumption of herbal products and drugs is increasing. They have various applications in adults and children due to the general belief about their safety. Since there is no toxicological evidence on the manna of *Alhagi pseudalhagi* and *Cotoneaster discolor*, their genotoxicity was evaluated in this study.

Manna, as a by-product of the activity of insects on young host plant organs, is a group of herbal compounds with medicinal and nutritional benefits. It is also of commercial value in Iranian traditional medicine market. The therapeutic characteristics of manna are attributed to its sacchariferous compound. The active substances of some manna have not yet been recognized and their chemical formula are not identified.[1]

Astragalus adscendens, Quercus brantii, Cotoneaster discolor, Echinops cephalotes, Alhagi pseudalhagi, Salix excelsa, and Saccharum officinarum are some of the vastly used Iranian manna among which Alhagi pseudalhagi (known in Iran as Taranjabin) and Cotoneaster discolor (known in Iran as Shir-Khesht) are the most important ones. These manna have been used as laxatives, antipyretics, and expectorants. They have also been employed in the treatment of hyperbilirubinemia in traditional medicine.[2,3] Common dosage for *A. pseudalhagi* is 100 mg/kg three times daily. For *C. discolor* on the other hand, it is 50 mg/kg for children and 150 mg/kg for adults.[3] *A. pseudalhagi* (a member of the Papilionaceae family) contains at least 80% sacchariferous ingredient based on its glucose content. Other ingredients of this manna include melezitose, saccharose, gum, mucilage, and different salts such as calcium, magnesium, sulfur, silicium, and aluminum salts.[4,5] Ethanolic extract of herbal products containing this manna has been shown to have inhibitory effects on cell growth and synthesis of cellular proteins, DNA, and RNA.[6]

The most important ingredient of *C. discolor* is mannitol. Other components include small amounts of hexose, fructose, glucose, saccharose, mucilage, and resin.[4,5] It is claimed that *C. discolor* protects the body against atomic radiations.[4] This manna has recently been introduced as an anticancer drug.[7,8]

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Genotoxicity tests are usually used as a way to identify DNA damages. Genotoxic factors can affect DNA directly or indirectly and cause DNA damage. This damage breaks in one or both strands of DNA. Rate of breaks depends on many factors such as the age of cells, type of cell line, and extracellular factors. In a genotoxic trial to determine DNA damage, highlighting probable mutations and abnormalities are crucial.[9,10]

From different methods of genotoxicity detection, comet assay, also known as single cell gel electrophoresis (SCGE), has been selected because of its high sensitivity and high quality in determination of DNA damages in single cells. This method was developed in 1984 by Ostling and Johanson as an electrophoresis method which made the direct observation of DNA damage possible for the first time. Each kind of eukaryotic cells could be used for genotoxic tests. $HepG_2$ cells (liver hepatocellular cells) were used in this study due to their active metabolic system and metabolic potency.[11]

As there is lack of toxicological evidence on these natural compounds, this research tried to increase our information about genotoxicity of *A. pseudalhagi* and *C. discolor* and to complete their safety profiles.

METHODS

The Roswell Park Memorial Institute (RPMI-1640), trypsin and fetal bovine serum (FBS) were purchased from PAA Company (Australia). Tris-hydrochloride (Tris-HCl), sodium hydroxide (NaOH), ethylenediaminetetraacetic acid (EDTA), and hydrogen peroxide (H_2O_2) were obtained from Merck Company (Germany). Low melting point (LMP) agarose, ethidium bromide, and trypan blue were purchased from Sigma Company (USA). Normal melting point (NMP) agarose was provided by Cinnagen Company (Iran).

Plant material

A. pseudalhagi (No. 10912, Department of Herbarium, Mashhad School of Pharmacy) manna was collected from northern Khorasan, Iran in summer 2008. *C. discolor* was purchased from Sobhan Pharmaceutical Company (Rasht, Iran). Both kinds of manna were standardized using quantitative determinations of mannitol by titration with 0.05 M iodine[3] and glucose by phenol-sulfuric acid test.[12] To prepare the stock solutions, these two kinds of manna and their sugars were dissolved in a medium [10.34 g of RPMI powder, 2 g of sodium bicarbonate ($NaHCO_3$), and up to 1 liter of water (H_2O)] with a pH of 7.4-7.6. The initial concentrations of these stock solutions were 250, 500, 250, and 400 mg/ml for *A. pseudalhagi*, *C. discolor*, glucose, and mannitol, respectively. The concentrations were determined based on the solubility of compounds in the medium. The solutions were filtered using 0.22 μm filters. They were then stored in refrigerator and used to prepare different concentrations for the comet assay.

Cell culture

In this study, $HepG_2$ cell line was obtained from National Cell Bank of Iran (NCBI). It was cultured in a medium of RPMI-1640 with 10% FBS and incubated under 5% carbon dioxide (CO_2) at 37°C. The cells were washed with phosphate buffer solution and harvested by trypsinization. The cell suspension was then centrifuged and the pellet was resuspended in the medium for viability determination (trypan blue exclusion).[13]

Alkaline comet assay

The stock solutions of *A. pseudalhagi* and *C. discolor* manna, glucose, and mannitol were added to the cell suspension (viability of more than 90%) to determine the genotoxicity of the substances under evaluation. Different concentrations of the substances were prepared from the stock solutions and incubated for 24 hours. Then, 300 μl of cells suspension (1×10^6 cells/ml) were mixed with 2 ml of 1% LMP agarose (37°C) and 100 μl of this suspension was placed on the slides that were precoated with 1% NMP agarose. Slides were covered with cover glasses and left for 5 minutes at 4°C. Cover glasses were then removed. For positive control slides, 200 μl of H_2O_2 (200 μM) were added to the negative cells for 20 minutes at 4°C and then were washed. During this period, negative controls (cells that were incubated in medium for 24 hours) and other slides were kept in a dark environment. The slides were then immersed in lysis solution [2.5 M sodium chloride (NaCl), 0.1 M EDTA, 0.01 M Tris-HCl, 0.2 M NaOH, 1% Triton x-100] at a pH of 10.0 for 40 minutes. Afterward, the slides were washed with distilled water to remove excess lysis solution. In the next step, the slides were placed in electrophoresis buffer (10 N NaOH, 200 mM EDTA) at pH > 13.0 for 40 minutes. Electrophoresis tank was filled with electrophoresis buffer and samples were electrophoresed for 40 minutes at 25 V with an electricity current adjusted to 300 mA.[14,15] After electrophoresis, the slides were removed and washed with distilled water to remove excess alkaline buffer. They were then placed in neutralization solution (0.4 M Tris-HCl) with a pH of 7.5 for 10 minutes. Each slide was subsequently covered with dye solution (20 μg/ml ethidium bromide) for 5 minutes and washed with distillated water. The comets were visualized under ×400 magnification using fluorescence microscopy with an excitation filter of 510-560 nm and

barrier filter of 590 nm.[14,15] All stages in comet assay were performed at 4°C in dark conditions and all solutions were cool.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) (Sigma Stat Version 3.1, Jandel Scientific Software, San Jose, California, USA) followed by post hoc test were used to analyze the data. The results were assumed to be significant if $p < 0.05$.

RESULTS

The amount of mannitol in *C. discolor* was estimated to be 42.547% by titration with 0.05 M iodine. On the other hand, the amount of glucose in *A. pseudalhagi* was estimated to be 86% using phenol-sulfuric acid test.

For each concentration, 3 independent experiments were performed with at least 30 comets per experiment. To evaluate the percentage of probable DNA damage promoted by the test substances, the extent of DNA damage was analyzed by computerized image analysis software (Comet Score, freeware). Tail length, percent of DNA in the tail, and tail moment were measured. Different concentrations of *A. pseudalhagi* (1, 2.5, and 5 µg/ml) and glucose solutions (0.25, 0.5, 1, 5, 10, and 25 mg/ml) were exposed to $HepG_2$ cell line for 24 hours. Their effects on DNA were evaluated by alkaline comet assay against control (Figures 1 and 3). Different concentrations of *C. discolor* (0.05, 0.5, 5, 50, 75, and 100 mg/ml) and mannitol solutions (5, 10, and 50 mg/ml) were similarly exposed to $HepG_2$ cell line for 24 hours and their effects on DNA were evaluated (Figures 2 and 4). Our findings showed that *C. discolor*, *A. pseudalhagi*, mannitol, and glucose were genotoxic at concentrations of 100 mg/ml, 5 µg/ml, 50 mg/ml, and 25 mg/ml, respectively.

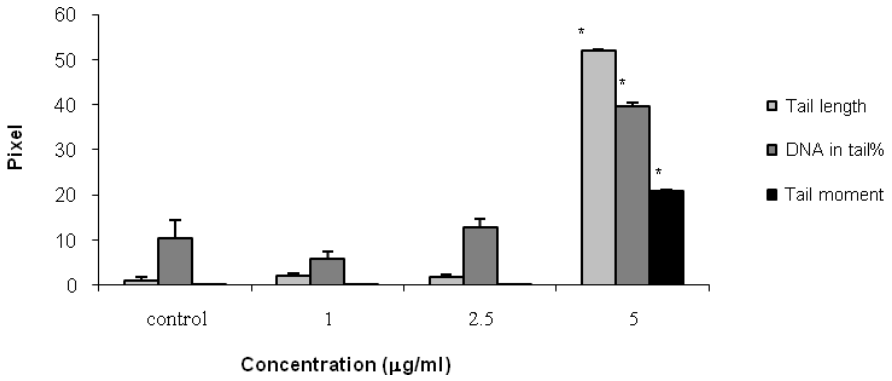

Figure 1. Genotoxicity parameters of Alhagi pseudalhagi by comet assay method (Different concentrations of A. pseudalhagi solution were exposed to $HepG_2$ cell line for 24 hours and their effects on DNA were evaluated by alkaline comet assay against control. Tail length (pixels), %DNA in tail, and tail moment (pixels) of 3 independent experiments, with at least 30 comet scores per experiment are represented as mean ± SD.) * $p < 0.05$

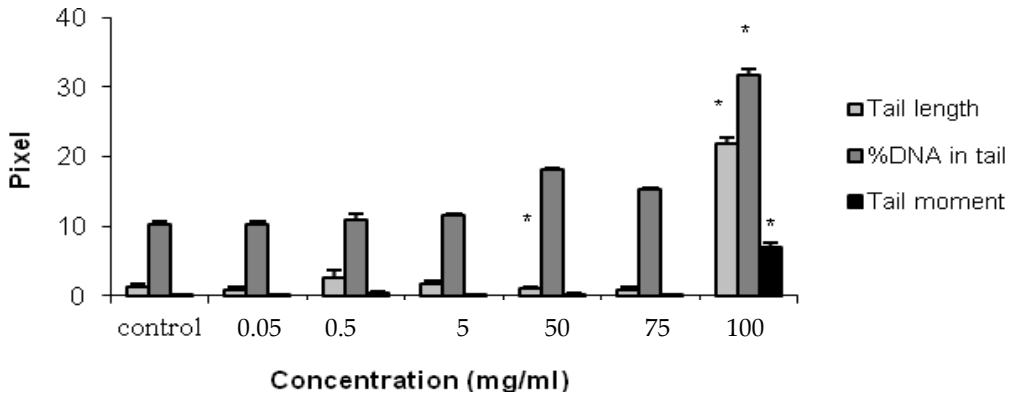

Figure 2. Genotoxicity parameters of *Cotoneaster discolor* by comet assay method (Different concentrations of *C. discolor* solution were exposed to $HepG_2$ cell line for 24 hours and their effects on DNA were evaluated by alkaline comet assay against control. Tail length (pixels), %DNA in tail, and tail moment (pixels) of 3 independent experiments with at least 30 comet scores per experiment are represented as mean ± SD.) * $p < 0.05$

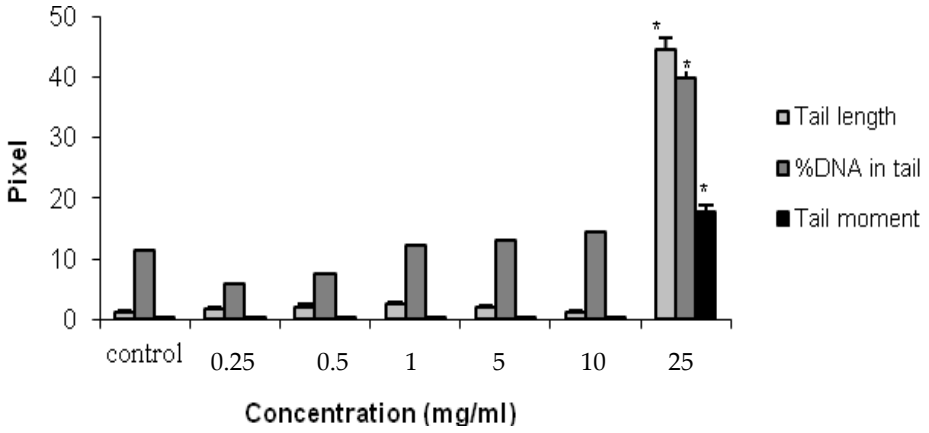

Figure 3. Genotoxicity parameters of glucose by comet assay method (Different concentrations of glucose solution were exposed to $HepG_2$ cell line for 24 hours and their effects on DNA were evaluated by alkaline comet assay against control. Tail length (pixels), %DNA in tail, and tail moment (pixels) of 3 independent experiments with at least 30 comet scores per experiment are represented as mean ± SD.) * $p < 0.05$

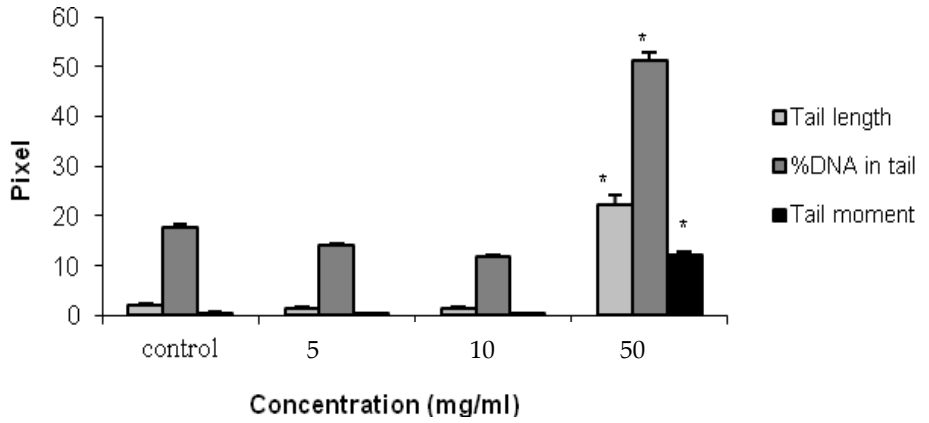

Figure 4. Genotoxicity parameters of mannitol by comet assay method (Different concentrations of mannitol solution were exposed to $HepG_2$ cell line for 24 hours and their effects on DNA were evaluated by alkaline comet assay against control. Tail (pixels), %DNA in tail, and tail moment (pixels) of 3 independent experiments with at least 30 comet scores per experiment are represented as mean ± SD.) * $p < 0.05$

DISCUSSION

C. discolor and *A. pseudalhagi* were studied here because of the increasing use of these kinds of medicinal manna, especially their use in treatment of jaundice in newborns, use of *C. discolor* in a pharmaceutical product (Billinaster drop from Sobhan Pharmaceutical Co., Rasht, Iran) in lower age groups, multiple drug applications in adults, and scarcity of information about their side effects including DNA profile. As *A. pseudalhagi* contains 50% glucose and *C. discolor* contains 80% mannitol, the effects of these two sugars were also studied as controls. Comet assay was used to evaluate DNA damage. This method is rapid, sensitive, easy to perform, inexpensive, and applicable to virtually any cell type from eukaryotic organisms. There have been several studies on the genotoxicity of plants. In one study, genotoxicity of *Acacia nilotica*, *Juglans regia,* and *Terminalia chebula* were evaluated by the comet assay and Vitotox test. The results showed comet assay to be more sensitive than other methods in evaluation of safeness of natural compounds.[16] In other studies, the potential mutagenic effects of Pothomorphe umbellata and *Physalis angulata* were assessed by the comet assay.[17,18] Figures 1-4 indicate that *A. pseudalhagi*, glucose, *C. discolor*, and mannitol were genotoxic at concentrations of 5 µg/ml, 25 mg/ml, 100 mg/ml, and 50 mg/ml, respectively. This study showed that these substances are safe at lower concentrations. Glucose was safe at a concentration of 10 mg/ml. This suggests that

the harm caused by *A. pseudalhagi* is not related to glucose and other unknown components may be responsible for this damage. We evaluated sugary compounds of this manna and it seems that they are safe. Glycoside and mucilaginous compounds of these compounds may have caused genotoxicity in these tests. Considering the usual doses of glucose, mannitol, and *C. discolor* that are used in Iranian traditional medicine, the use of these products may be safe, but the high amounts of *A. pseudalhagi* (ten times more than the common dosage) is not recommended. It is noteworthy that almost all substances may cause DNA damage in high concentrations. DNA damage in the alkaline comet assay may be impermanent and hence may not necessarily result in mutation. Therefore, other genotoxicity tests need to be performed on these substances. *A. pseudalhagi* samples which are sourced from the other parts of the country should also be checked by similar tests.

CONCLUSIONS

Taking *C. discolor* and *A. pseudalhagi* in concentrations less than 100 mg/ml and 5 μg/ml, respectively is safe. Evaluation of DNA damage due to mannitol and glucose showed that their concentrations of respectively 50 mg/ml and 25 mg/ml are genotoxic. Therefore, the harmful effects of non-sugary components of the tow mentioned kinds of manna might be considered in the toxicity caused by these compounds.

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